MAPT as pathogenic risk factor for Parkinson's Disease

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Resumo

A doença de Parkinson é uma doença neurodegenerativa com uma etiologia complexa, incluindo fatores genéticos e ambientais. Em todo o mundo, mais de seis milhões de pessoas sofrem com a doença. A mesma é considerada uma sinucleinopatia, caracterizada pela acumulação de corpos de Lewy e degeneração de neurônios dopaminérgicos principalmente no mesencéfalo.

O gene codificante da proteína tau associada aos microtúbulos está presente no cromossomo 17q21.31. Juntamente com outros 18 genes, está localizado em um num desequilíbrio de ligação de 952 kb existente em dois haplótipos. O haplótipo em orientação direta é chamado H1 e o inverso, H2. O haplótipo MAPT mais comum, H1, tem sido associado às tauopatias, bem como à doença de Parkinson. Também foi demonstrado que o polimorfismo de nucleótido único rs8070723 do haplótipo H1 está associado a um risco aumentado a doença. A proteína Tau possui 6 isoformas, uma expressão mais elevada de da sua isoforma 4R tau pode influenciar o desenvolvimento de proteinopatias como tauopatias e sinucleinopatias.

Várias alterações em funções celulares estão associadas à patogênese da doença de Parkinson idiopática. O haplótipo de risco MAPT pode aumentar a suscetibilidade para neurotoxinas e fatores ambientais. Este projeto visa investigar se o risco de polimorfismo de nucleótido único rs8070723 do haplótipo H1 de MAPT pode potencializar neurotoxicidade causada por estressores bioquímicos associados à doença de Parkinson.

As “human fetal neural progenitor cells” derivadas do mesencéfalo de espécimes com diferentes haplótipos de MAPT foram tratadas com rotenona, epoxomicin, wortmannin e H_2O_2. A susceptibilidade às toxinas foi analisada através do ensaio de lactato desidrogenase.

Observamos que as células com o haplótipo H1/H1 apresentaram maior susceptibilidade aos estressores bioquímicos quando comparadas às células com o haplótipo H2/H2. Além disso, a expressão de tau analisada 1 e 2 semanas após a diferenciação mostrou que principalmente três isoformas de Tau foram expressas: 2N3R, 1N4R e 0N3R. No entanto, são necessárias mais experimentações para confirmar esses dados.

Palavras-chave: Doença de Parkinson, MAPT, hmNPC, Sinucleinopatia, Neurotoxicidade

Abstract

Parkinson's disease is a neurodegenerative disorder with a complex etiology, including genetic and environmental factors. Worldwide, over six million people suffer from the disease. It is considered a synucleinopathy, characterized by accumulation of Lewy bodies and degeneration of dopaminergic neurons primarily in the midbrain.

The coding gene of microtubule associated protein tau is present on chromosome 17q21.31. Together with 18 other genes it is located in a 952 kb linkage disequilibrium that exists in two haplotypes. The haplotype in direct orientation is named H1 and the inverted, H2. The most common *MAPT* haplotype, H1, has been linked to tauopathies as well as Parkinson's disease. It has also been shown that the single nucleotide polymorphism rs8070723 of the H1 haplotype is associated with an increased risk for the disease. Tau protein has 6 isoforms, a higher expression of the 4R tau isoform might influence the development of proteinopathies such as tauopathies and synucleinopathies.

Several alterations of cellular functions are associated with the pathogenesis of idiopathic Parkinson's disease. The *MAPT* risk haplotype might increase susceptibility for neurotoxins and environmental factors. This project aims to investigate if the risk single nucleotide polymorphism rs8070723 of the *MAPT* haplotype H1 might potentiate neurotoxicity caused by biochemical stressors associated with Parkinson's disease.

Human fetal neural progenitor cells derived from the midbrain of specimens with different *MAPT* haplotypes were treated with rotenone, epoxomicin, wortmannin and H_2O_2. Toxin susceptibility was analysed via the lactate dehydrogenase assay.

We observed that cells with the H1/H1 haplotype displayed a higher susceptibility towards biochemical stressors when compared to cells with H2/H2 haplotype. In addition, the expression of tau analysed 1 and 2 weeks post differentiation showed that mainly three Tau isoforms were expressed: 2N3R, 1N4R and 0N3R. However, further experiments are necessary to confirm this data.

Key Words: Parkinson's disease, MAPT, hmNPC, Synucleinopathy, Neurotoxicity

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Abbreviations

ALP	Autophagy- lysosomal pathway
ANOVA	Analysis of variance
AO	Antioxidants
DAPI	4',6-Diamidino-2-Phenylindole
DMSO	Dimethylsulfoxide
GWAS	Genome wide association studies
H_2O_2	Hydrogen peroxide
hmNPC	Human midbrain neural progenitor cells
IPS	Idiopathic Parkinson syndrome
Lambda PP	Lambda Protein Phosphatase
LDH	Lactate dehydrogenase
MAPT	Gene of microtubule associated protein tau
MAPT	Microtubule associated protein tau
MMP	Mitochondrial Membrane potential
M-PER	Mammalian Protein Extraction Reagent
PBS	Phosphate buffered saline
PD	Parkinson disease
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
TBST	Tris-buffered saline (TBS) and Polysorbate 20
UPS	Ubiquitin proteasome system
UTC	Untreated control

1 Introduction

1.1 Parkinson's disease

Over six million people suffer from Parkinson disease (PD), or idiopathic Parkinson syndrome (IPS), placing this disease as the second most common neurodegenerative disease worldwide [6], [39]. PD is a complex neurodegenerative disorder with genetic and environmental factors as its etiology [13]. Advanced age is an important factor to this disorder; the population over 65 years of age is 1–2% affected by PD and it is estimated that the number of cases will rise in the following years [57].

The main characteristic of this disease is the degeneration of dopaminergic neurons in the substantia nigra pars compacta and its classical parkinsonian motor symptoms [30], [50]. Most patients are only diagnosed with PD after the manifestation of motor symptoms, which include resting tremor, slowness of voluntary movement, rigidity and postural instability [31], [57]. At this point, up to 80 % of dopaminergic cells in the nigro-striatal system are estimated to be lost [57]. Apart from this, Parkinson's patients also manifest non-motor dysfunctions, some of which precede the motors symptoms by more than a decade [30]. IPS's non motor symptoms include anosmia, REM sleep behavioural disorder, depression and forms of dementia might also be observed [69]. IPS is a progressive degenerative illness of the human nervous system that manifests itself clinically after the pathology has already reached an advanced stage [7].

Lewy Neurites (LNs) and Lewy Bodies (LB) are intracytoplasmic inclusions with a spindle or a globular form, respectively [14]. Their presence lead to the loss of dopaminergic neurons in the substantia nigra pars compacta and other brain regions [31]. The major component of LNs and LB is aggregated α-synuclein, a presynaptic protein expressed also in dopaminergic neurons which function involves the maintenance of synaptic vesicles necessary for neurotransmission [14], [60]. The α-synuclein aggregates are often present years before the diagnosis of PD [7]. The Lewy Bodies, in the pre-symptomatic stages of PD, outset in the *medulla oblongata*, in the *pontine tegmentum* and in the *olfactory bulb/anterior olfactory nucleus* [69]. When it spreads to the midbrain, the firsts motor symptoms start to occur [7]. In the end stage, when the disease reach the neocortex, a variety of clinical manifestations such as dementia and psychosis start to occur [7], [69].

The etiology of PD can be characterized by two different causes: inherited, which affects between 10 to 15 % of PD patients and non- inherited, also called idiopathic Parkinson syndrome (IPS) [76].

Mutations in several genes have already been associated to autosomal dominant (*SNCA*, *LRRK2* and *VPS35*) and autosomal recessive (*PARK-2*, *PARK-7*, DJ-1 and *PINK-1*) forms of PD [13], [76]. Mutations in the *SNCA* gene, which express the α-synuclein protein, are associated with premature cognitive impairment and might cause symptoms of early dementia [20]. On the other hand, the autosomal recessive genes PARK-2 and PINK-1 tend to cause severe motor symptoms with less cognitive impairment [20].

Cellular impairments such as oxidative stress and mitochondrial, proteasomal and autophagic dysfunctions are suggested to contribute to IPS [40]. In combination with genetic variants, environmental factors might increase the risk for Parkinson's disease [4]. Multiple genome-wide association studies (GWAS) have identified small risk loci that might play a crucial role for the development of neurodegenerative disorders such as PD [13]. Interestingly, beside *SNCA*, *MAPT* was the second most important risk factor [13].

Until this day, there is no effective treatment to slow or stop the progression of PD [69]. The standard therapy with drugs that increase intracerebral dopamine concentrations or stimulate dopamine receptors, such as L-Dopa, a dopamine precursor, only target the correction of motor disturbances [30], [69]. The development of more effective drugs is a major goal of Parkinson's disease research, therefore comprehension of underlying pathophysiological mechanisms are very important [6].

1.2 *MAPT*, tau and the idiopathic Parkinson syndrome

Neurodegenerative diseases are classified on the basis of their protein substrates [55]. The two major groups are the synucleinopathies, linked to α-synuclein, Lewy bodies and Lewy neurites and the tauopathies, related to microtubule-associated protein TAU [54]. Idiopathic Parkinson's syndrome is the most common synucleinopathy. However, both *SNCA* and *MAPT* genes are known to contribute with PD susceptibility [3], [78], [82].

The tau protein was discovered in 1975 and it was one of the first microtubule- associated proteins to be described [37]. Tau is a natively unfolded protein and associated to the cytoskeleton of the nervous system [12], [32]. Aggregates of tau filaments and its deposition in neuronal or glial fibrillary inclusions define the pathological characteristics of tauopathies [1].

Human tau protein is encoded by the *MAPT* gene, located on chromosome 17q21 and comprises 16 exons [33]. Alternative splicing of the exons 2, 3 and 10 generate six different isoforms of the protein (Figure 1) [37]. The isoforms differ in the number of amino acids inserted at the N-terminal region (N) and the amount of microtubule repeats (R), creating the following isoforms: 2N4R, 1N4R, 0N4R and 2N3R, 1N3R, 0N3R [20]. Tau isoforms length range from 352 to 441 amino acids (36.8 to 45.9 kDa) [47]. mRNAs missing exon 10 generate the 3R isoform meanwhile mRNAs including exon 10 encode the 4R tau [47]. In the developing brain, the 3R tau isoforms are dominant, while in an adult brain all six tau isoforms can be found [11]. The ratio of 4R:3R tau expression in a healthy adult brain is approximately equal [34]. Meanwhile the ratio between the 0N, 1N and 2N is 37:57:9 [53]. Some mutations may affect the splicing of tau mRNA, resulting in increased expression of the 4R tau isoforms compared to 3R tau isoforms [35]. Imbalance between tau isoforms might be related to tauopathies [1].

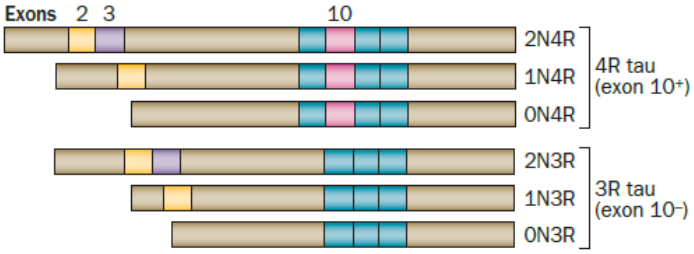

Figure 1: Tau mRNAs after splicing. The six tau isoforms are generated by alternative splicing and differ from each other in the number of amino acids inserted at the N-terminal region (N) and the amount of repeats (R). Adapted from [77].

Phosphorylated tau is necessary to control the equilibrium between tau and microtubules [59]. Although tau phosphorylation is a normal and reversible process, an increased MAPT expression can disrupt the structure of microtubules by the accumulation of misfolded and abnormal phosphorylated tau protein, which in turn may lead to tauopathies [10], [12].

The *MAPT* gene possesses an ancestral inversion polymorphism which generates a 1.8 Mb region of linkage disequilibrium (LD) [10], [49]. This inversion leads to different haplotypes with distinct orientations. H1 which has direct orientation, is evolutionary dynamic and composed of a variation of single nucleotide polymorphisms (SNPs) [46], [49]. The second haplotype, H2, has an inverted orientation of ~970 kb. It is uncommon and relatively invariant (Figure 2) [77]. The LD of the H2 haplotype might have been the result of a single event or by a chromosome rearrangement or by a limited intermixing with a predating population [21].

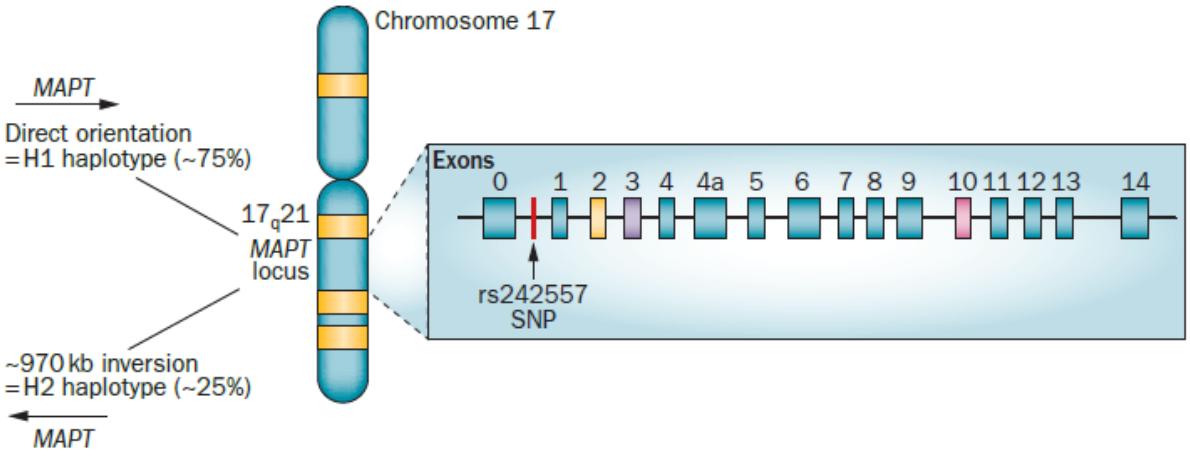

Figure 2: *MAPT* gene locus and its haplotypes. Tau protein is encoded by the MAPT locus on chromosome 17. The haplotypes H1 and H2 differ from each other through single nucleotide polymorphisms (SNP) and are among other genes within complete linkage disequilibrium. The H2 haplotype has an inversion polymorphism. Adapted from [77].

The H1 haplotype, and most specifically the H1c variant, has been associated not only to an increased risk of neurodegenerative disorders such as tauopathies but also with an higher risk of Parkinson's disease [27], [49], [54], [73]. It was also suggested that the H2 haplotype has a protective effect on the development of PD [65]. Furthermore, the H1c haplotype increases the relative levels of 4R tau isoform due to promoting higher exon 10 incorporation [46]. The *MAPT* gene was identified via GWAS as the second most important risk factor for IPS (Figure 3) [19].

Figure 3: Distinct SNPs on the H1 haplotype associated with different neurodegenerative disorders. SNPs within the intronic regions of the *MAPT* gene [10]. The marked SNP rs8070723 is linked to a higher risk for idiopathic Parkinson syndrome [19]. Adapted from [9].

In neurological disorders an interaction between pathogenic proteins is common, resulting in a disturbance of their normal function through a downstream activity modulation [59]. A genetic relation between the proteins α-synuclein and tau in IPS was previously reported [59].

The risk genotypes *MAPT* and *SNCA* when expressed individually have a marginal effect on PD development, however, the interaction between those two genes may have a higher influence on this event [24]. Studies suggest that the links between tau and α-synuclein are

functional and pathological [73]. α-synuclein influences tau phosphorylation and its fibrillization, inducing a risk factor for IPS [73]. IPS progression can be stimulated by the formation of hybrid α-synuclein and tau oligomers which are able to accelerate the oligomerization process [59].

1.3 Environmental factors related to the idiopathic Parkinson syndrome

Another important risk factor for the development of PD are environmental influences. Factors as pesticide exposure, head injury, use of β-blockers among others, were already referred as risks factors [30]. On the other hand, tobacco smoking, coffee drinking were considered protective factors to PD development [30]. Induced cell death by apoptotic processes involving a cascade of events such as oxidative stress, inflammation, mitochondrial dysfunction and excitotoxicity were also suggested as risks factor for PD progress [48].

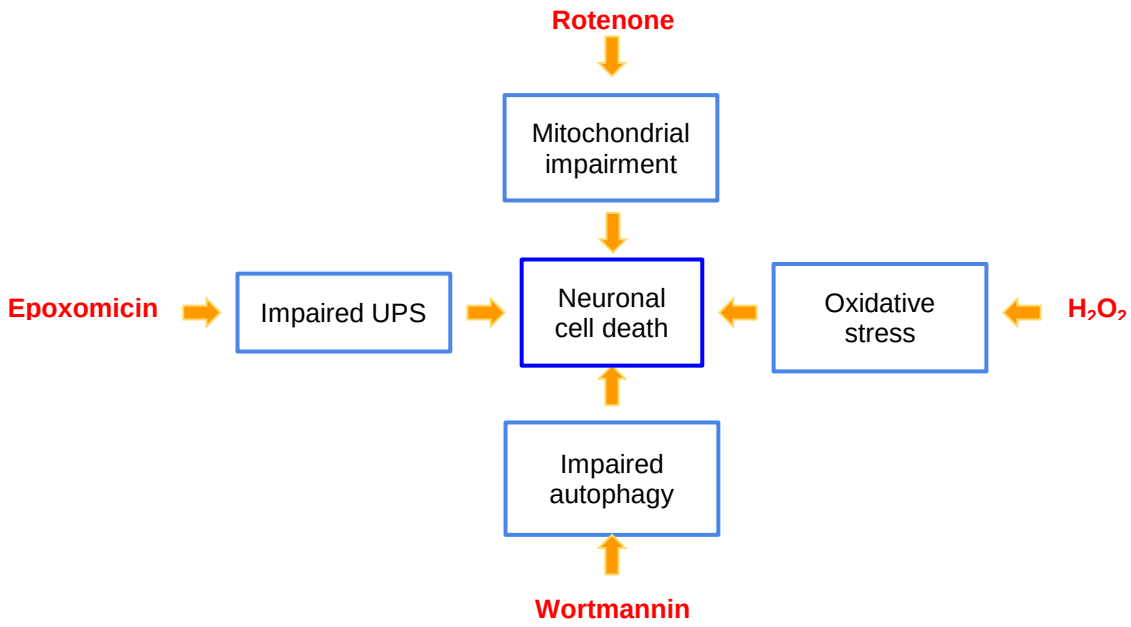

Figure 4: Examples of cellular dysfunctions observed in the idiopathic Parkinson syndrome (clear blue box) caused by its respective neurotoxins (red letters) that eventually lead to cell death (dark blue box).

During this master thesis we monitored the effects of four neurotoxins: epoxomicin, wortmannin, rotenone and H_2O_2 in order to mimic the environmental influence in human midbrain derived neural progenitor cells with the *MAPT* haplotypes in homozygosity.

1.3.1 Mitochondrial inhibition and rotenone

Also, environmental factors are associated with IPS, as an example is the exposure to pesticides [2]. Rotenone is a compound commonly used as a pesticide which acts as an inhibitor of the mitochondrial complex I [2], [70]. Rotenone treatment also leads to an increased ROS production [26]. Since dopaminergic neurons already have a higher ROS generation as a result from dopamine metabolism, they are more vulnerable to oxidative stress and therefore toxins like rotenone [17]. Therefore, to induce mitochondrial impairment, rotenone was employed in our cell model.

1.3.2 Proteasome inhibition and epoxomicin

In eukaryotic cells, the degradation of abnormal proteins is situated in the cytoplasm, nucleus and the endoplasmic reticulum by the ubiquitin-proteasome system (UPS) [48]. A failure in this system is suggested to play a role in PD's etiology [48]. When the UPS fails, for example due to defects in the 20S proteasome, it can lead to accumulation and aggregation of proteins. Protein accumulation and aggregation of α-synuclein is a hallmark of PD and may cause neurotoxicity [41]. One example of this failure is the possible formation of the Lewy bodies in dopaminergic neurons of the substantia nigra pars compacta, a characteristic of PD [41]. The chosen toxin to inhibit the UPS in our cell lines, epoxomicin, is a specific proteasome inhibitor which binds to the 20S proteasome, thus inhibiting irreversibly four catalytic subunits.

1.3.3 Autophagy inhibition and wortmannin

Another protein and organelle degradation pathway is the autophagy-lysosomal pathway (ALP) [18], [79]. Autophagy is characterized by the degradation of cytoplasmic proteins and organelles via lysosomal enzymes aiming the recycling of cellular components or the prevention of the accumulation of those structures [84]. To sequester cytosolic compartments, organelles, proteins and lipids, autophagosomes are formed. The autophagosomes fuse in later stages with lysosomes or late endosomes, which contain stored degrading enzymes [18].

Since aggregated proteins can't penetrate the proteasome barrel, in IPS, the aggregated α-synuclein from the Lewy bodies are degraded via ALP, while its soluble forms might be degraded via UPS [79]. Wortmannin disturbs the autophagosome formation via inhibition of the assembly of the double membrane, which eventually forms the autophagic vesicle [84].

Hence, wortmannin was used to achieve autophagic impairment from the start of the autophagy process.

1.3.4 Oxidative stress and H_2O_2

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide radicals, are generated by the mitochondria during mitochondrial respiration as a by-product of oxidative phosphorylation [16].

Although the brain is very sensitive to oxidative damage, about 20% of the oxygen resources of the organism are consumed by it [15]. ROS production in the neurons is extremely high due to a high oxygen output rate, a limited availability of antioxidant and a high amount of fatty acids that are easily unsaturated [15], [39]. ROS oxidize lipids, DNA, RNA and proteins either directly or indirectly [15]. A balance between ROS production and degradation is necessary to the homeostasis [16]. Cell damage and possible death can be promoted by a disrupted balance which allows ROS accumulation and oxidative stress [16]. H_2O_2 was employed in our cell model to induce oxidative stress.

1.4 Cellular model to investigate the idiopathic Parkinson syndrome

In order to investigate the etiology of IPS and other neurodegenerative disorders, different human cellular models are frequently used. To explore the molecular mechanisms of IPS, human neuronal cells are the best choice [56].

To this master thesis, the fetal human midbrain neural progenitor cells (hmNPCs) were used. HmNPCs are isolated from aborted fetuses after 10-14 weeks after fertilization followed by preparation of region specific cell lines. These tissue- specific precursors are able to self-renewal and can be cultured for months without closing their potential of differentiation into neurons and glial cells [45], [66]. Furthermore, hmNPC are able to differentiate into dopaminergic neurons, this ability creates a promising tool to IPS research, either for the improvement of cellular models to the disease etiology study or for cell therapies for Parkinson's patients [45], [80]. HmNPCs are a valuable reference model for neuronal cells derived from induced pluripotent stem cells too. But most importantly, hmNPC give us the opportunity to investigate and compare cell lines with different *MAPT* haplotypes.

2 Objectives

Aim of the present master thesis is to explore different mechanisms by which the risk *MAPT* haplotype, H1/H1, may contribute to the development of IPS. Therefore, different hmNPC lines with the *MAPT* haplotypes H1/H1 and H2/H2 for the risk SNP rs8070723, were submitted to neurotoxic treatments aiming to induce cellular dysfunctions, such as induction of oxidative stress, mitochondrial and proteasome impairment and autophagy inhibition.

The same cell line with the same haplotypes mentioned above, were also differentiated for distinct periods of time aiming to chart the expression of the tau isoforms.

During this project the following points were investigated:

1. Induction of neurotoxicity to hmNPC.
2. Comparison of toxic impact between different *MAPT* genotypes.
3. Comparison between the expression of the tau isoforms after 1 and 2 weeks of cell differentiation in different hmNPC haplotypes via Western blot.

3 Materials and Methods

3.1 Human midbrain neural progenitor cell culture

3.1.1 Ethics

All experiments performed in this master thesis were approved by the ethics committee of the University of Leipzig and the Technical University of Munich, Germany in accordance with all state and federal guidelines. The chosen cell lines were prepared from aborted fetuses posterior to the mother's consent.

3.1.2 Preparation of hmNPCs

HmNPCs have been generated as described by Moon *et al*. [45]. To guarantee tissue specificity, the cells were analysed for dopamine transporter expression by flow cytometry in order to characterize them as mesencephalic: at least 0.5% of the cells had to be positive for dopamine transporter expression for mesencephalic classification. This result was confirmed by the expression of tyrosine hydroxylase via Western blot, as described by Milosevic *et al*. [43], [44]. Furthermore, the expression of markers of dopaminergic neurons was assessed by RT-PCR [45].

3.1.3 Cultivation of hmNPCs

The cells were cultured in Dulbecco's Modified Eagle's Medium/Ham's F12 medium (HyClone, GE Healthcare, Little Chalfond, UK) containing 2 % B27® supplement minus AO (Thermo Fisher Scientific, Darmstadt, Germany), 0.1 % gentamicin (Thermo Fisher Scientific, Darmstadt, Germany), 0.1 % of the mitogens rhu-EGF and rhu-bFGF-2 (Peprotech, Hamburg, Germany) and 0.1 % tocopherol and tocopheryl acetate (Sigma-Aldrich, Taufkrichen, Germany). Cells were cultured as monolayers in cell t75 culture flasks (NUNC; Sigma) coated with poly-L-ornithin (Sigma-Aldrich, Taufkrichen, Germany) and human fibronectin (Millipore, Merck, Darmstadt, Germany) in a humidified incubator at 37°C with a reduced oxygen atmosphere of 3 %.

3.1.4 Coating

For coating, a 15 µg ml-1 poly-L-ornithin solution was applied to cell t75 culture flasks (NUNC; Sigma-Aldrich, Taufkrichen, Germany) and 24 or 96 wells plates and incubated for

3h at 37°C. The culture dishes were washed with 1x PBS, then incubated with a 4 µg ml-1 human fibronectin solution (Millipore, Merck, Darmstadt, Germany) for 3h at 37°C. The flasks and plates were washed with PBS and stored at -80°C until use.

3.1.5 Expansion of hmNPCs

For expansion of the hmNPC lines, 6 million cells were seeded out and the medium was exchanged completely twice a week. The cells were passaged when a confluency of at least 80 % was achieved. For passaging, the cells were detached with 1x Accutase® (PAN biotech, Aidenbach, Germany) by incubation for 30 minutes at 37°C. Cells were spun down at 1500 rpm, re-suspended in fresh medium and counted. For experiments, cells were seeded into pre- coated culture plates of different sizes.

3.1.6 Differentiation of hmNPCs

For cell differentiation, a neurobasal medium (Invitrogen) supplemented with 2 % B27® supplement minus AO (Thermo Fisher Scientific, Darmstadt, Germany), 1x GlutaMAX (Thermo Fisher Scientific, Darmstadt, Germany) and 100 µM dibutiryl c-AMP (Sigma-Aldrich, Taufkrichen, Germany) was used. The hmNPC were differentiated for 8 days prior to experimental read out.

3.2 Toxin application

To perform the toxin assays cell lines were seeded in 96 well plates at a density of 30.000 to 35.000 cells per well in expansion medium until it reached at least 80 % confluency, around 3 to 5 days after seeding. After achieving the expected confluency, the medium was changed to differentiation medium and the cells were differentiated for 8 days. At day 6 or 7 of differentiation, depending on the applied toxin (Table 1), the medium was replaced by 100 µl of new differentiation medium containing the diluted toxins (Figure 5).

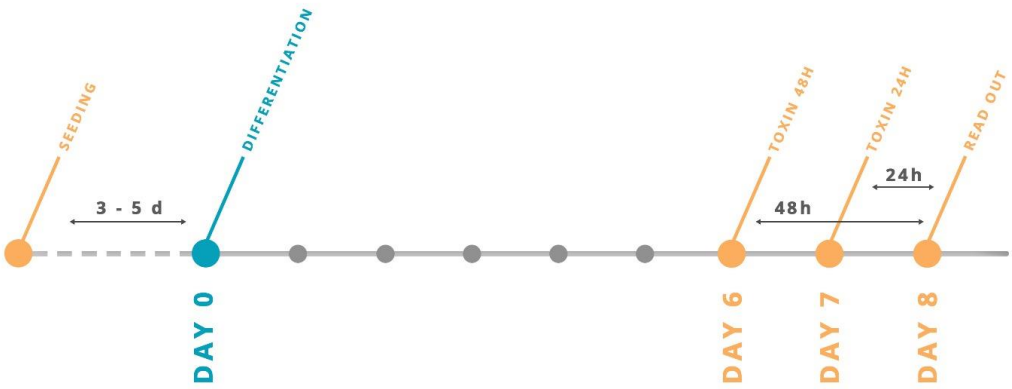

Figure 5: Timeline of toxins application.

Rotenone (Sigma-Aldrich, Taufkrichen, Germany) and epoxomicin (ApexBio technology LLC, Houston, USA) were received as powder meanwhile wortmannin (Sigma-Aldrich, Taufkrichen, Germany) was ordered as a solution. The toxins were dissolved in dimethylsulfoxide (DMSO) to the maximal soluble concentration as listed in Table 1. All stock solutions were stored at -80 °C. H_2O_2 (Sigma-Aldrich, Taufkrichen, Germany) did not require a first dilution, it was kept as ordered in a 30 weight percent solution in water, corresponding to a 9.1 M concentration, it was stored in the dark at 4 °C and the dilutions for the cell treatments were prepared freshly before use. The rotenone aliquots were only used once upon thawing.

Substance name	Dissolved in and dilution	Treatment concentration	Treatment duration	Source	Catalogue number
Rotenone	Dissolved in DMSO to 100 mM; diluted further in medium.	50 µM and 100 µM	24 hours	Sigma Aldrich	R8875
Epoxomicin	Dissolved in DMSO to 10 mM; diluted further in medium.	1 µM and 10 µM	48 hours	ApexBio technology LLC	A260
Wortmannin	Dissolved in DMSO to 10 mM; diluted further in medium	5 µM and 10 µM	48 hours	Sigma Aldrich	WS3144
H_2O_2	Stock at 30 % or 9,1 M; diluted further in medium	20µM and 40µM	24 hours	Sigma Aldrich	16911

Table 1: Toxins and their features used for experiments.

The rotenone stock was dissolved in the medium by the addition of 2 % DMSO, an additional UTC with 3 % DMSO was used for this toxin. The epoxomicin and wortmannin stocks were diluted 1:100 in culture medium, not exceeding an end concentration of 1 % DMSO. For this untreated control (UTC), DMSO without compound was diluted 1:100 resulting in 1 % DMSO in medium. Depending on the compound's toxicity, the chemicals were applied at day 6 and 7 of differentiation and incubated for 48 and 24 hours, respectively. The cell viability was then determined at day 8 for all treatment conditions.

3.3 Cell toxicity assay - LDH assay

The acute cell toxicity was assessed by measuring the release of NADH into the medium. In the chosen protocol LDH turns over pyruvate to lactate by using up NADH. Therefore, the consumption of NADH is proportional to LDH available in the medium.

The reaction buffer consisted in 100 mM of pyruvate (Sigma-Aldrich, Taufkrichen, Germany), 10 mM NADH (Sigma-Aldrich, Taufkrichen, Germany), in 80 mM Tris/HCL (Carl Roth, Karlsruhe, Germany) and 200 mM NaCl (Sigma-Aldrich, Taufkrichen, Germany) with pH 7.2. The positive control was lysed by lysis buffer (Promega, Mannheim, Germany) for 40 min. 30 µl supernatant of samples, positive and medium only (blank) wells were transferred to a new clear 96 well plate. 70 µl of the reaction buffer was added directly before measurement. The absorbance was measured immediately with the CLARIOstar microplate reader (BMG Labtech, Ortenberg, Germany) at 340 nm for the absorption of NADH and 420 nm as reference measurement for 10 x 30 sec cycles. The blank-corrected values from cycle 3 to 7 were then used for linear regression and the calculation of the curves steepness as they correlate to the amount of released LDH. The lysis control represents the maximal possible LDH release correlating with maximal toxicity. All values were normalized to the lysis control.

3.4 Western blotting

The cell lines with different differentiation times were detached with 1x Accutase® (PAN biotech, Aidenbach, Germany) by incubation for 30 minutes at 37°C, spun down at 1500 rpm for 5 min and re-suspended in 100 µl of M-PER (Thermo Fisher Scientific, Darmstadt, Germany) with protease and phosphatase inhibitors (Roche, Mannheim, Germany) and maintained at -80°C until use. A BCA assay (Thermo Fisher Scientific, Darmstadt, Germany) was performed in order to determine the protein concentration of the samples. An internal protein standard was used as an internal control for the Western blot analysis.

3.4.1 Lambda Protein Phosphatase (λ PP)

Lambda Protein Phosphatase is a Mn2+ dependent protein phosphatase used to release phosphate groups from phosphorylated serine, threonine and tyrosine residues in proteins. 20 µg of the above mentioned samples were treated with the Lambda Protein Phosphatase (Lambda PP) Kit by New England BioLabs (Frankfurt, Germany), following the manufacturer's instructions. 20 µg of protein, MPER buffer, a master mix of 10x PMP Buffer & 10 mM $MnCl_2$ and 1µl of Lambda Protein Phosphatase were mixed and incubated at 30°C for 3 h without shaking. The loading buffer, 4X laemmli, was added and the samples were boiled at 75°C for 10 minutes. Unless indicated otherwise, samples were kept on ice.

3.4.2 SDS PAGE and blotting

Proteins were separated by SDS PAGE. Therefore, samples were loaded in a bis-tris 10 % gradient sodium dodecyl sulfate (SDS) polyacrylamide gel (BioRad, Hercules, USA). Two ladders were loaded into the gel, 4 µl of ProSieve protein ladder (Biozym, Hessisch Oldendorf, Germany) and a 2 µl of tau ladder. Electrophoresis was performed using the following settings: 75 V for 10 minutes, then 160 V for 70 min. Proteins were then transferred onto a PVDF membrane (BioRad, Hercules, USA) by wet transfer at 35 V for 2 hours at 4°C.

3.4.3 Blocking and Antibody staining

The membrane was fixed with 3,75 % PFA (Sigma) and blocked with a solution of 3x rotiblock (Carl Roth) in TBST. Posteriorly, the membranes were incubated with the following antibodies: Polyclonal Rabbit Anti-Human Tau (DAKO, Agilent, Santa Clara, USA) and Anti-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) Antibody (Millipore, Merck, Darmstadt, Germany), respectively, at concentrations of 1:3000, followed by incubation with Peroxidase labelled anti rabbit IgG (Vecto laboratories, Maravai Lifesciences, San Diego, USA) at a 1:5000 concentration, and read out at Licor Odyssey FC (Lincoln, USA).

	Antibody	Staining	Dilution	Company	Catalogue number
Primary antibody	Polyclonal Rabbit Anti-Human Tau	Total tau	1:3000	DAKO	A002401-2
Primary antibody	Anti-Glyceraldehyde-3-Phosphate Dehydrogenase	GAPDH	1:3000	Milipore	Abs16
Secondary antibody	Anti rabbit IgG	Peroxidase labelled anti rabbit IgG	1:5000	Ab Vector	PI-1000

Table 2: Details from primary and secondary antibodies used at Western Blotting.

3.5 Statistical analysis

With the objective of comparing the toxicity assays from different cell lines and their *MAPT* haplotypes, the data acquired from the LDH assay had to be normalized to the respective control based on the following equation:

$$x = \frac{T - C}{100 - C} \cdot 10$$

T is related to the percentage of LDH release when treated with the toxin and C the LDH release from the UTC. Its respective p values were calculated with the two-way analysis of variance (ANOVA) at the program GraphPad Prism 7 (GraphPad Software, San Diego, USA). The data is represented as mean ± SEM.

4 Results

4.1 Comparison of the toxin susceptibility of the different haplotypes

For each condition, 3 biological repeats with 4 technical repeats for the H1/H1 and H2/H2 *MAPT* haplotypes were performed in hmNPC lines and statistically analysed by the two-way ANOVA test. For each *MAPT* haplotype, 2 different hmNPC lines were used, excluding the ones used in the H_2O_2 toxicity assay. Due to a change in protocol only one cell line per haplotype was used in the analysis.

4.1.1 Rotenone treatment of the different haplotypes

The cells were incubated with 50 µM and 100 µM rotenone for 24 h. The acute toxicity measured by LDH release was significantly higher in hmNPC lines bearing the H1/H1 haplotype when compared to hmNPC lines of the H2/H2 after 50 µM and 100 µM rotenone application, p- value < 0.005 and < 0.0001 respectively. The cell toxicity at 50 µM rotenone was 9 % in the H1/H1 haplotype compared to -2 % to the H2/H2. While at the 100 µM concentration the difference between the haplotypes was 19 % in H1/H1 and 2 % in H2/H2. These results are shown in Figure 6.

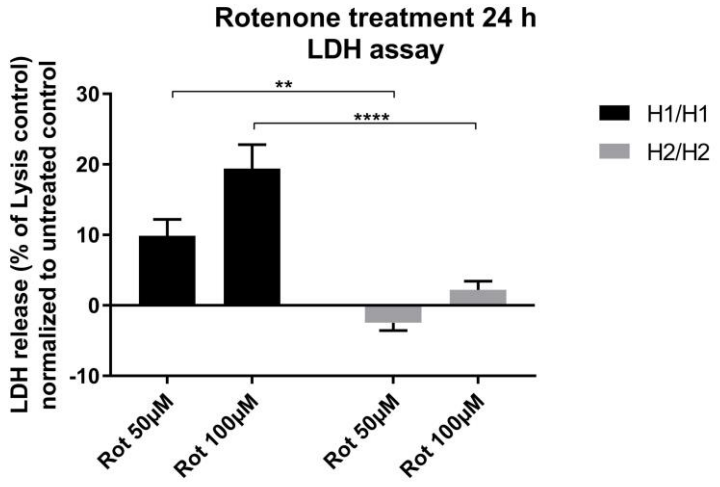

Figure 6: Effect of 50 µM and 100 µM rotenone exposure for 24h in hmNPC lines with *MAPT* haplotypes H1/H1 and H2/H2. LDH release analysis was normalized to untreated controls to determine toxicity. Graph represent mean +SEM. n=8. Significance was calculated by two-way ANOVA: **p< 0.005 and ****p< 0.0001.

4.1.2 Epoxomicin treatment of the different haplotypes

The hmNPC lines were incubated with epoxomicin in concentrations of 1 µM and 10 µM for 48 h. The LDH assay revealed a highly significant difference of epoxomicin induced acute toxicity between hmNPC lines. In concentrations of 1 µM and 10 µM the H1/H1 haplotype showed an increase of LDH release when compared to the H2/H2 p-value $p< 0.0001$ and $p< 0.0001$, respectively. After application of 1 µM epoxomicin the LDH release for the H1/H1 haplotype was 46 % and 17 % of the H2/H2 compared to the untreated control. The 10 µM epoxomicin treatment induced the release of LDH of 49 % in the H1/H1 haplotype and 19 % in the H2/H2 cell lines compared to the untreated control. Shown in figure 7.

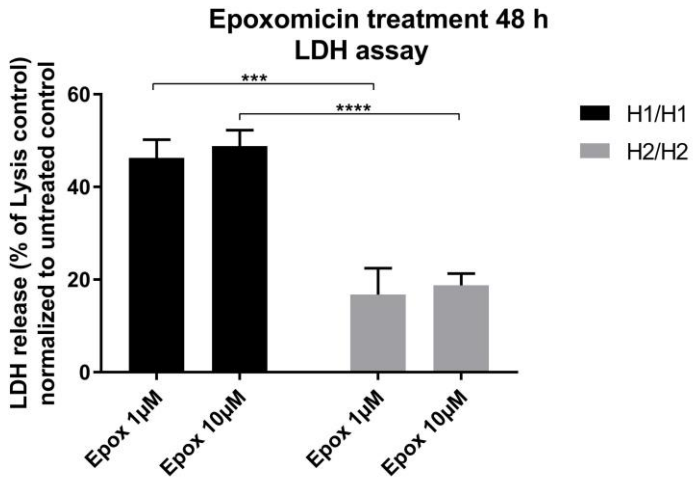

Figure 7: Effect of 1 and 10 µM epoxomicin exposure for 48h in hmNPC lines with *MAPT* haplotypes H1/H1 and H2/H2. LDH release analysis was normalized to untreated controls for determination of toxicity. Graph represent mean +SEM. n= 6. Significance was calculated by two-way ANOVA: ***$p< 0.0001$ and ****$p< 0.0001$.

4.1.3 Wortmannin treatment of the different haplotypes

The hmNPC cell lines were incubated with5 µM and 10 µM wortmannin for 48 h. No significant differences could be observed with the LDH assay between the haplotypes. The cell toxicity at 5 µM wortmannin was 14 % in the H1/H1 haplotype compared to 7 % to the H2/H2. In the 10 µM concentration the difference between the haplotypes was 19 % to H1/H1 and 13 % to H2/H2. As demonstrated in Figure 8.

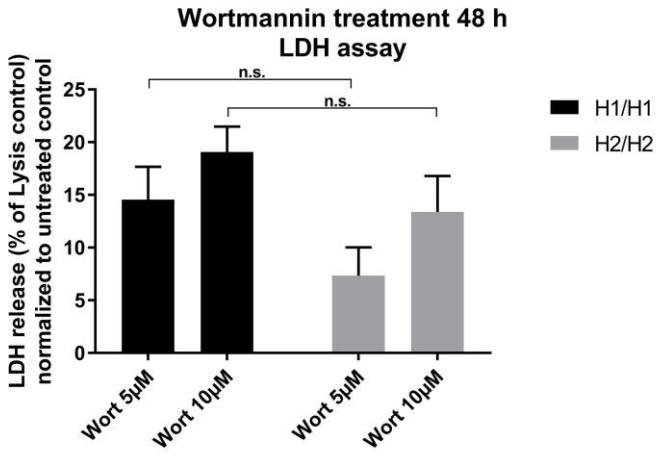

Figure 8: Effect of 5 μM and 10 μM wortmannin exposure for 48h in hmNPC lines with *MAPT* haplotypes H1/H1 and H2/H2. LDH release analysis was normalized to untreated controls to determine toxicity. Graph represent mean +SEM. n= 7. Significance was calculated by two-way ANOVA; both values were nonsignificant.

4.1.4 H_2O_2 treatment of the different haplotypes

The hmNPC cell lines were incubated with H_2O_2 at concentrations of 20 μM and 40 μM for 24h. After treatment with 20 μM H_2O_2 both haplotypes showed a small statistical significance. Meanwhile, after the treatment with a higher H_2O_2 concentration (40 μM), no significant differences were observed, as demonstrated at the Figure 9. The 20 μM concentration induced a cell toxicity of 65 % and 38 % respectively for the H1/H1 and H2/H2. Meanwhile the treatment with 40 μM H_2O_2 showed a cell toxicity of 70 % and 64 % to H1/H1 and H2/H2 haplotypes.

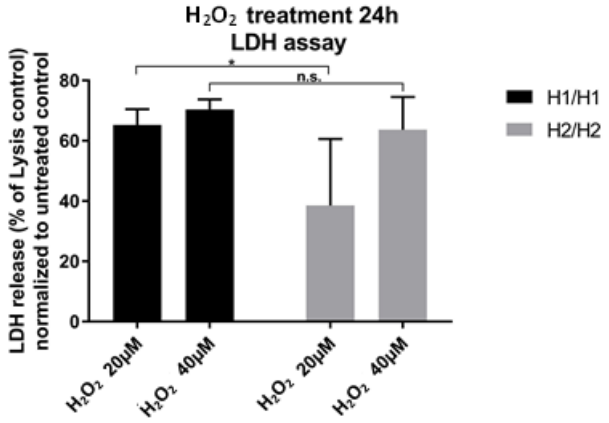

Figure 9: Effect of 20 and 40 μM H_2O_2 exposure for 24h on hmNPC lines with *MAPT* haplotypes H1/H1 and H2/H2. LDH release analysis was normalized to untreated controls to determine toxicity. Graph represent mean +SEM. n=4. Significance was calculated by two-way ANOVA: *p< 0.05

4.2 Expression of tau isoforms in different haplotypes

In order to study the expression of different tau isoforms, we developed Western blot protocols for TAU isoforms involving treatment with Lambda phosphatase (λ PP). The Western Blots were performed with different *MAPT* haplotypes (H1/H1, H2/H1 and H2/H2) and cell lines one and two weeks post differentiation. The protein was loaded in triplicates. The representative pictures of the Western blots are shown in figures 10 and 11.

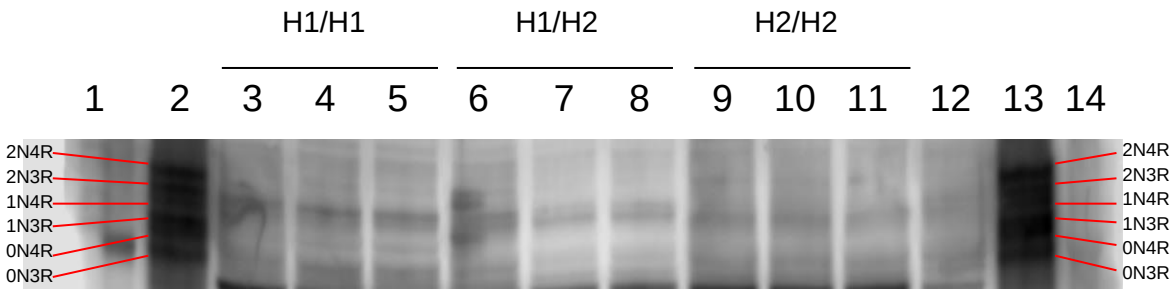

Figure 10: Western blot analysis of tau after 1 week of differentiation. Electrophoresis with the different hmNPC lines with distinct *MAPT* haplotypes: H1/H1 (3-5), H2/H1 (6-8) and H2/H2 (9-11); internal protein standard (12); tau ladder (2 and 13) and ProSieve protein ladder (1 and 14). The six recombinant human brain tau isoforms are shown in the first lanes, molecular masses of 36.8, 39.7, 40.0, 42.6, 42.9, and 45.9 kDa, respectively. All hmNPC samples were treated with λ PP before the assay.

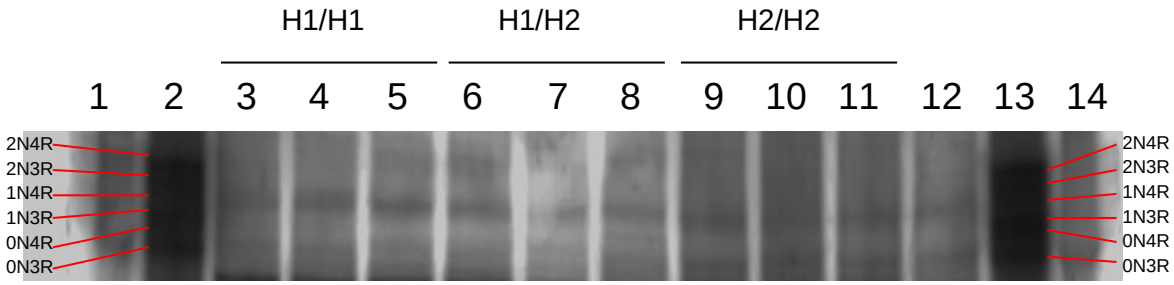

Figure 11: Western blot analysis of tau after 2 weeks of differentiation. Electrophoresis with the different hmNPC lines with distinct *MAPT* haplotypes: H1/H1 (3-5), H2/H1 (6-8) and H2/H2 (9-11); internal protein standard (12); tau ladder (2 and 13) and ProSieve protein ladder (1 and 14). The six recombinant human brain tau isoforms are shown in the first lanes, molecular masses of 36.8, 39.7, 40.0, 42.6, 42.9, and 45.9 kDa, respectively. All samples were treated with λ PP.

Analysis of the dephosphorylated tau by λ PP treatment enabled identification of the individual tau isoforms present in each sample. The protocol of the TAU isoform western blots was in progress at the end of this master thesis. Therefore, the results presented are not optimal and cannot be further evaluated. Briefly, in both blots we were able to observe mainly the expression of three tau isoforms: 2N3R, 1N4R and 0N3R.

5 Discussion

5.1 Toxin treatment of the different haplotypes

The H1 haplotype of the MAPT gene has been associated to an increased risk of Progressive Supranuclear Palsy and as the second most common risk factor for PD [19], [27].

HmNPC lines bearing different *MAPT* genotypes create a promising tool for IPS studies and allow the opportunity to consider invariable unmodified human neural cell lines with distinct genetic backgrounds [45], [58]. Although there is no evidence about the pathogenic mechanism behind neurodegeneration in IPS patients, some cellular and molecular dysfunctions such as mitochondrial and ubiquitin- proteasome dysfunctions, inhibition of autophagy and oxidative stress were already related to the disease's etiology [5], [48].

We treated hmNPC lines with different *MAPT* haplotypes with rotenone, epoxomicin, wortmannin and H_2O_2 to mimic the cellular and molecular dysfunctions mentioned above for 24 or 48h to measure the acute toxicity between the genotypes. It was hypothesized that the *MAPT* risk haplotype would suffer a higher impact after the toxins treatment, developing a greater susceptibility towards environmental toxins. After 8 days of differentiation and appliance of toxins for 24 or 48h, hmNPC were submitted to LDH assay revealing an increased toxicity of rotenone and epoxomicin of the H1/H1 compared to the H2/H2 genotypes. The treatment with wortmannin, however, showed no significant statistical difference between the genotypes.

5.1.1 Rotenone treatment

As mentioned before, mitochondrial dysfunction is associated with IPS [53], based on that we treated the cells with rotenone, a mitochondrial complex I inhibitor. We incubated the cells with the toxin for 24h at the concentrations of 50 and 100µM. The results showed a significant difference in toxicity between the haplotypes.

Taking into account the difference in cell toxicity via LDH assay, the chosen time point for the read out was accurate. We could also observe that just a small fraction of cells revealed a toxic feature after rotenone treatment. The hmNPC cells are unaltered human fetal neural progenitor cells derived from the midbrain. Moon *et* Schwarz characterized these cells and found between 25 and 30 % of NPC differentiate into the dopaminergic phenotype. Dopaminergic neurodegeneration caused by the mitochondrial complex I impairment is

believed to happen preferably via oxidative stress than mediated by a bioenergetic defect [60]. Considering that rotenone also increases ROS levels [26], the death of the dopaminergic subpopulation seems likely. On the contrary, the manifestation of mitohormesis [71], already reported for small concentrations of this toxin [85] might have contributed to cell survival of more robust neuronal subpopulations and glial cells.

Taking into account the unspecificity of the LDH assay, an evaluation of mitochondrial function in neurons of the different haplotypes could be assessed by a mitochondrial potential assay (MMP). The MMP assay monitors changes to cells capacity to generate ATP by oxidative phosphorylation, indicating cell health or damage [51]. In addition, double immunofluorescence for dopaminergic and apoptotic markers could address the question, which cells undergo apoptosis after rotenone treatment.

5.1.2 Epoxomicin treatment

The cells were treated with 1 and 10 µM epoxomicin for 48h, a proteasome inhibitor, in order to test the influence of proteasomal dysfunction on the different hmNPC haplotypes [42]. epoxomicin inhibits the catalytic function of proteasome, thus impairing the UPS [42]. The LDH assays showed a significantly higher LDH release at the H1/H1 haplotype when compared to the H2/H2. It is suggested that the inhibition of the proteasome reduces the activity of the complex I and II of the mitochondria which influences the electron transport chain, the main source of ROS [68]. As consequence of the treatment with epoxomicin, the proteasome induces mitochondrial dysfunction and a higher level of ROS.

5.1.3 Wortmannin treatment

The cells were treated with the autophagy inhibitor wortmannin at 5 and 10 µM for 48 h in order to impair the autophagic-lysosomal pathway [84]. The differences between the haplotypes in wortmannin induced toxicity were not significant for any of the dosages in the given time frame.

One consequence of the inhibition of autophagy is the accumulation of impaired organelles, proteins and other cell components [18]. This might lead to increased oxidative stress, mitochondrial dysfunction and protein aggregation, ultimately leading to cell death [83]. However these mechanisms may need a longer incubation time to develop their harmful potential, therefore we suggest that the time of the treatment (48h) might not have been long enough to activate the autophagy inhibition. A constant inhibition of autophagy over 72 hours or even longer [75] might affirm more outstanding differences in toxicity for the haplotypes.

5.1.4 H_2O_2 treatment

Focusing the induction of oxidative stress, the cells were treated with 20 and 40 µM for 24h [68]. Even though we could observe a higher toxicity for the H1/H1 haplotype when compared to the H2/H2, both haplotypes developed a very high toxicity and a small statistical difference was only observed after treatment with the lower concentration of H_2O_2.

The decrease of viable cells observed after H_2O_2 treatment can provide evidence of its highly potential of toxicity [25]. We speculate that in the LDH assay the 24h exposure time and the elected concentrations might have been too high to reveal the genotypes differences. The sensitivity for differences in LDH release might be increased by lower concentrations or reduced exposure time. Another fact that should be taking into consideration is the instability of H_2O_2 [74]. Even though the solutions were prepared immediately before application, a high variability of toxic impact is demonstrated by the error bars.

5.1.5 Increased susceptibility of the H1/H1 haplotype to toxins and Parkinson's disease

After treatment with rotenone acute toxicity assessed by LDH assay after rotenone treatment was markedly higher in the H1/H1 MAPT haplotype compared to H2/H2 , thus suggesting a higher susceptibility. The H1 haplotype increased risk of Parkinson's disease [49], together with the dopaminergic neurodegeneration by the complex I impairment [60], suggests that the combination of MAPT genotype and biochemical stressor could possibly generate a higher risk for PD. The results of the epoxomicin treatment also indicate a higher susceptibility of the H1/H1 genotype by proteasome inhibition in hmNPC in the H1/H1 MAPT haplotype.

At this time point, the reason for these higher susceptibilities is rather speculative. It could be related with an increased tau expression from the H1 haplotype, notably the 4R isoform [46], [62]. It has already been suggested that the aggregation of α-synuclein could be enhanced by an increased tau concentration due to its elevated expression levels [22], [37]. The oxidative damage resulted from rotenone exposure might also be related to the aggregation of α-synuclein [60]. In vivo experiments have already described that rotenone induces aggregated forms of α-synuclein in the substantia nigra, due to oxidative stress [60].

The proteasome inhibitor, epoxomicin, binds to the 20S proteasome. Defects on this subunit can exceed the capacity of degradation of the UPS aggresome leading to the aggregation of

poorly degraded proteins [41]. This defect will promote protein aggregation in dopaminergic neurons [41]. Additionally, disruptions in proteasome function might influence the production of ROS and the oxidative damage [72]. Aggregated α-synuclein could inhibit proteasomal and autophagic pathway by binding to the proteasome and avoiding the formation of the autophagosome [63], [83]. In addition, the overexpression of tau could disturb the axonal transport of vesicles and organelles in neurons and cause defects in mitochondria [52], [64], [65].

In summary, these data suggest that combination of the H1 MAPT risk haplotype with the cellular impairments caused by treatment with rotenone or epoxomicin increases the susceptibility of the development of Parkinson's disease.

5.2 Expression of tau isoforms in different haplotypes

The tau protein has 6 isoforms in the adult human brain; these isoforms are generated by alternative splicing and differ from each other by the number of inserts in its N-terminus and by the number of repeats in the C-terminal [11]. Since the different isoforms are expressed in distinct phases during the development, each of them is related to a physiological role [1]. In early stages of brain development and its embryonic phase the 3R tau is the primarily expressed isoform, while in the adult brain all six tau isoforms are available [11]. 3R tau isoforms do not bind as tightly as 4R tau isoforms to microtubules, further, tau phosphorylation interfere negatively to the amount of tau that binds on the C-terminal [11]. An attachment of 3R to 4R monomer or polymer is possible to happen, decreasing 4R tau association, however, at normal circumstances, unless the 3R binding was irreversible a decrease at the amount of 4R would not happen [1].

We could observe on our Western Blots a constant presence of the 1N4R tau isoform. In humans, the 1N4R tau isoform is the most abundant [80]. Another isoform constantly expressed in our assays was the human fetal tau protein isoform, the 0N3R. This isoform is expressed during fetal development [59] with the expression peak at mid gestation period and until birth [29].

6 Conclusions

This thesis had the purpose of comparing the susceptibility of hmNPC lines with different *MAPT* haplotypes, after exposure of toxins which provoke cellular dysfunctions associated with IPS. In addition, the development of protocols for evaluation of 3R and 4R tau isoforms to study tau isoform expression over distinct differentiation times in the different *MAPT* haplotypes was anticipated.

The acute cell toxicity, assessed via LDH assay, revealed a higher toxin susceptibility for the H1/H1 genotype compared to the H2/H2 after treatment with rotenone and epoxomicin a complex I inhibitor and a proteasome inhibitor, respectively. These results suggest that the H1 risk-haplotype might be involved in mitochondrial and proteasomal dysfunction to IPS development. Further studies with autophagy inhibitor wortmannin and the inductor of oxidative stress H_2O_2 should be performed.

The hmNPC lines, after one or two weeks of differentiation seem to express the 3R tau isoform more abundantly than the 4R isoform, however the assay is still in progress. Here, we suggest additional experiments. After optimization at the tau WB, we propose inducing toxicity in hmNPC at different times of differentiation, followed by LDH measurement and Western Blot to the different tau isoforms, in order to compare the 4R:3R after stress.

7 References

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